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(54) Title: METHOD OF INHIBITING PADGEM-MEDIATED INTERACTIONS USING AN INHIBITOR COMPRISING A 2,6 SIALIC ACID COMPONENT

(57) Abstract

The invention relates to a method of inhibiting (reducing or preventing) the interaction or adhesion of a PADGEM-bearing cell with a cell bearing a PADGEM ligand by contacting the PADGEM-bearing cell with an inhibitor comprising a 2,6-linked sialic acid component. By the method of the present invention, it is possible to inhibit the interaction of a PADGEM-bearing cell, such as a platelet or endothelial cell, with a cell bearing a PADGEM ligand, such as a white blood cell (leukocyte) by contacting the cell with an inhibitor comprising a 2,6-linked sialic acid residue. The invention further relates to inhibitors useful in the present method.

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METHOD OF INHIBITING PADGEM-MEDIATED INTERACTIONS USING AN INHIBITOR COMPRISING A 2,6 SIALIC ACID COMPONENT

Background of the Invention

The LECCAMs or selectins, including Mel-14 antigen, LAM-1 (LECAM1), ELAM-1 (LECAM2), and PADGEM (LECAM3), are a newly recognized class of cellular adhesion molecules that are characterized structurally by the presence of a lectin-like domain, an epidermal growth factor-like domain, a variable number of cysteine-rich repeats related to those found in a family of complement regulatory proteins, a transmembrane domain, and a short cytoplasmic tail (Osborn, L., Cell 62: 306 (1990)). These cell adhesion molecules are thought to function in the adhesion of leukocytes to endothelial cells.

For example, the Mel-14 antigen and its human analog, LAM-1, which are present on the surface of lymphocytes, are thought to be involved in the targeting of lymphocytes to endothelial cells within high endothelial venules (Siegelman, M.H. et al., Science, 243: 1165-1172 (1989); Tedder, T.F. et al., J. Exp. Med., 170: 123-133,(1989)). ELAM-1 (endothelial-leukocyte adhesion molecule) has been shown to mediate the interaction of endothelial cells with neutrophils and monocytes (Bevilacqua et al., Science 243: 1160-1165 (1987)). The cell adhesion molecule PADGEM (platelet activation dependent

granule-external membrane protein), present on the platelet surface, has been implicated in the adhesion of stimulated platelets to neutrophils and monocytes through specific recognition sites present on the neutrophils and monocytes. This interaction is calcium dependent (Larsen, E. et al., Cell 59: 305-312 (1989); Geng et al., Nature 343: 757-760 (1990); Hamburger, S.A. and McEver, R.P., Blood, 75: 550-554 (1990)).

The platelet activation dependent granule-external membrane protein, PADGEM, has been cloned and has a typical LECCAM structure, with a lectin domain, an epidermal growth factor domain, nine complement binding repeat domains, a transmembrane domain, and a cytoplasmic domain (Johnston, G.I. et al., Cell, 56: 1033-1044, (1989)). PADGEM, which is also referred to as GMP-140, CD62, or LECAM3, is found on the endothelial cell surface as well as on the surface of platelets. PADGEM is also found in megakaryocytes, which are the precursors of platelets. (Beckstead et al., Blood 67: 285-293 (1986)). In endothelial cells, PADGEM is stored as a component of the Weibel-Palade bodies (Bonfanti, R. et al., Blood 73: 1109-1112, (1989)), and in platelets, it is stored as a component of the alpha granule membrane. Following granule exocytosis, PADGEM is expressed on the cell surface (Hsu-Lin, S.C. et al., J. Biol. Chem., 259: 9121-9126, (1984); Berman, C.L. et al., J. Clin. Invest., 78: 130-137 (1986); McEver, R.P. and Martin, M.N., J. Biol. Chem., 259: 9799-9804, (1984);

Stenberg, P.E. et al., J. Cell Biol., 101: 880-886 (1985); Hattori, R. et al., J. Biol. Chem., 264: 7768-7761 (1989)). In contrast, certain cytokines stimulate the synthesis of ELAM in endothelial cells, leading to its expression on the plasma membrane after 4-6 hours (Bevilacqua, M.P. et al., Proc. Natl. Acad. Sci. USA, 84: 9238-9242 (1987)).

Both PADGEM and ELAM-1 are lectins that bind to lineage-specific carbohydrates on the surface of certain leukocytes (Larsen et al., Cell 63: 467-474 (1990); Lowe et al., Cell 63: 475-484 (1990)). The data suggest both ligands have a common Le^x core. Thus, surface carbohydrate structures could contribute to the specificity of the cell-cell interactions mediated by PADGEM and ELAM-1.

Summary of the Invention

The present invention relates to a method of inhibiting (reducing or preventing) the interaction or adhesion of a PADGEM-bearing cell with a cell bearing a PADGEM ligand by contacting the PADGEM-bearing cell with an inhibitor comprising a 2,6-linked sialic acid component under conditions whereby adhesion or interaction is inhibited. By the method of the present invention, it is possible to inhibit the interaction of a PADGEM-bearing cell, such as a platelet or endothelial cell, with a cell bearing a PADGEM ligand, such as a white blood cell (leukocyte) by contacting the cell with an inhibitor comprising a 2,6-linked sialic acid residue. Using

this method, it is possible to inhibit the adhesion of leukocytes (e.g., neutrophils, monocytes) to platelets and/or endothelial cells.

As shown herein, antibodies directed against the CD15 cell surface antigen inhibit the interaction of PADGEM-bearing cells (e.g. platelets and COS cells bearing PADGEM) with leukocytes (e.g. neutrophils, monocytes). Furthermore, as shown herein, Lacto-N-fucopentaose (LNF III), a complex carbohydrate which comprises a Lewis x (Le^x) core component and is recognized by CD15 antibodies, inhibits the binding of stimulated platelets to neutrophils. Additional data suggests that inhibition by LNF III is specific for PADGEM-mediated adhesion and implicates a Le^x core structure in inhibition. The data suggest that PADGEM can interact with a Le^x core (e.g., Galβ1-4(Fucα1-3)GlcNAc) and that inhibitors comprising a Le^x core can inhibit PADGEM-mediated adhesion.

In addition, neuraminidase treatment of HL60 cells decreased PADGEM-mediated binding, suggesting that sialic acid may also be part of the PADGEM ligand. Although ELAM-1 has also been shown to recognize a sialyated Le^x (SLe^x) core structure, sialyl 2,3 Le^x and related structures, as shown herein, a distinct PADGEM ligand is implicated. In particular, the demonstration of specific inhibition of PADGEM-leukocyte interaction by a NANA 2,6-specific lectin directed against Neu5Acα(2,6)Gal or Neu5Acα(2,6)GalNAc (e.g., sialyl 2,6 Gal) suggests

that an α 2,6-linked sialic acid is an important feature of the PADGEM ligand. The invention further relates to inhibitors of PADGEM-mediated cell-cell interaction. Inhibitors of PADGEM-mediated cell-cell interaction useful in the present method comprise a 2,6-linked sialic acid (NeuAc) component. For example, useful inhibitors can comprise Neu5Acα2,6Galβ1-4(Fucα1-3)NAcGlc) or other α2,6 sialylated α(1-3) fucosylated lactosamines or polylactosamines. An inhibitor comprising a 2,6-linked sialic acid component can further comprise a CD15 immunoreactive component, such as Le^x or all or a portion of Le^x or LNF-III.

Brief Description of the Drawings

Figure 1 illustrates the effects of a panel of anti-leukocyte antibodies on the interaction of neutrophils and activated platelets. The percent adherence corresponds to the percentage of cells with two or more adherent platelets under the assay conditions.

Figure 2 illustrates the inhibitory effects of 80H5 monoclonal antibody on the interaction of thrombin-stimulated platelets with monocytes (Mono), neutrophils (PMN), U937 cells (U937) and HL60 cells (HL60). The height of the bar indicates the percent binding or percentage of cells with two or more adherent platelets in the absence of antibody (black bars) or in the presence of antibody (hatched bar).

Figure 3 illustrates the effect of the concentration of anti-CD15 antibody 7C3 on the inhibition of the binding of activated platelets to neutrophils. The percent adherence corresponds to the percentage of cells with two or more adherent platelets under the assay conditions.

Figure 4 illustrates the inhibition of adherence of ^{111}In-labelled U937 cells to COS/PADGEM cells by monoclonal antibody 80H5 as assayed by counting ^{111}In activity.

Figure 5 illustrates the results of a FACS analysis of the interaction of U937 cells with phospholipid vesicles containing purified PADGEM. A histogram of log red fluorescence is given on the X axis and cell number is given on the Y axis. U937 binding to phospholipid vesicles without PADGEM (dotted line), to phospholipid vesicle containing PADGEM (dashed and dotted line), and to phospholipid vesicles containing PADGEM in the presence of anti-CD15 antibody (solid line) is shown.

Figure 6 illustrates the inhibitory effects of LNF isomers, LNF I (open squares), LNF II (closed circles), and LNF III (closed squares), on the interaction of activated platelets and neutrophils.

Figure 7 illustrates the effects of LNF isomers LNF I (open squares), LNF II (closed circles), and LNF III (closed squares), on the interaction of HL60 cells with COS cells expressing PADGEM. Standard errors of triplicate experiments are given by the error bars.

Figure 8 illustrates the results of an adhesion assay in which the adhesion of HL60 cells to CHO-PADGEM (hatched bar) or CHO-ELAM (black bar) cells treated with A. ureafaciens, V. cholerae or Newcastle disease virus neuraminidase was monitored. HL60 cell binding to neuraminidase-treated cells is recorded as a percent of binding observed with untreated control cells.

Figure 9 illustrates the inhibition of adhesion of HL60 cells to CHO-PADGEM (open circles) or CHO-ELAM (filled circles) cells by purified PADGEM as a function of PADGEM concentration (μg/ml).

Figure 10 illustrates the effect of Sambucus nigra lectin on the adhesion of HL60 cells to CHO-PADGEM (filled circles) or CHO-ELAM (open circles) as a function of the concentration (μg/ml) of Sambucus nigra lectin. Binding of HL60 cells to lectin-treated cells is recorded as a percent of the HL60 cell binding to untreated control CHO-PADGEM or CHO-ELAM cells.

Detailed Description of the Invention

The present invention relates to a method of inhibiting (reducing or preventing) the interaction of a cell bearing PADGEM with its target ligand by contacting the cell with an inhibitor comprising a 2,6 linked sialic acid component. The invention further relates to a method of inhibiting (reducing or preventing) the interaction or adhesion of endothelial cells or platelets with leukocytes (i.e.,

white blood cells), especially with nonlymphocytic leukocytes such as neutrophils and monocytes, by contacting the endothelial cells or platelets with an inhibitor comprising a 2,6-linked sialic acid component. For example, the interaction of a PADGEM-bearing cell, such as a platelet or endothelial cell, with a cell bearing a PADGEM ligand (e.g., neutrophils and monocytes) can be inhibited by contacting the PADGEM-bearing cell with an inhibitor comprising a 2,6-linked sialic acid residue.

Identification of a PADGEM-ligand

To identify the natural PADGEM ligand on neutrophils and monocytes, a series of monoclonal antibodies prepared against a variety of leukocytes and derivative cell lines was surveyed to identify those that bind to structures on leukocytes, but do not bind to platelets, and those that also inhibit the interaction of activated platelets with leukocytes. Of the antibodies surveyed, only those directed against CD15 met these criteria. As shown in Example 3, antibodies directed against the CD15 cell surface antigen inhibit the interaction of PADGEM-bearing cells (e.g. platelets and COS cells bearing PADGEM) with leukocytes (e.g. neutrophils, monocytes). The observation that antibodies to CD15 blocked the interaction of activated platelets with neutrophils, monocytes, HL60 cells, and U937 cells suggested that CD15 on the white cell surface may be directly involved with or located in close proximity

to the PADGEM ligand. In fact, several lines of evidence indicate that the PADGEM ligand actually shares structural features with CD15 positive structures.

CD15 is a carbohydrate antigen associated with glycolipids, glycoproteins, and proteoglycans (Kobata and Ginsburg, J. Biol. Chem., 244: 5496-5502 (1969); Yang and Hakomori, J. Biol. Chem., 246: 1192-1200 (1971); Huang et al., Blood, 61: 1020-1023 (1983); Skubitz and Snook, J. Immunol., 139: 1631-1639 (1987); Christiansen and Skubitz, Blood, 71: 1624-1632 (1988)). This antigen is defined by a branched-chain oligosaccharide, LNF III (Huang et al., Blood, 61: 1020-1023 (1983). This pentasaccharide and its related isomers, LNF I and LNF II, are abundant in human milk (Kobata and Ginsburg, J. Biol. Chem., 244: 5496-5502 (1969)). In addition to its distribution on neutrophils and monocytes, this carbohydrate is a marker for adenocarcinoma of the lung, colon and stomach, and for certain forms of lymphoma (Hall and Ardenne, J. Clin. Pathol., 40: 1298-1304 (1987); Sanders et al., J. Pathol., 154: 255-266 (1988)). The CD15 antigen is a component of glycolipids (Fukuda et al., J. Biol. Chem., 260: 1067-1082 (1985)), glycoprotein O-linked oligosaccharides (Carlsson et al., J. Biol. Chem., 261: 1287-12951986), and glycoprotein N-linked oligosaccharides (Fukuda et al., J. Biol. Chem., 260: 12957-12967 (1985)) on human granulocytes. Specific glycoproteins present on the leukocyte surface have been shown to carry CD15 antigens and include LFA-1,

Mac-1, gp150,95 (CD11/CD18), and CR1 (the C3b receptor) as well as proteins with molecular weights of 105,000 and 145,000 and a phosphotyrosine-containing protein of about 180,000 (Albrechtsen and Kerr, Br. J. Haematol., 72: 312-320 (1989); Skubitz et al., J. Immunol., 141: 4318-4323 (1988)). Although these proteins may be distributed among many vascular cell types, only on specific leukocytes, such as neutrophils and monocytes, do their structures include the complex carbohydrate LNF III.

Thus, the observation that three different anti-CD15 monoclonal antibodies inhibit the binding of activated platelets to monocytes and neutrophils, and that the distribution of CD15 on different vascular cells is parallel to the distribution of the PADGEM ligand, suggests that the PADGEM ligand and CD15 antigen are related. The demonstration in Example 3 that CD15 antibodies also inhibit the interaction of monocye-like cells (U937) with COS cells transfected with PADGEM or with phospholipid vesicles containing purified PADGEM emphasizes the specificity of the anti-CD15 antibody inhibition for PADGEM mediated adhesion.

As shown in Example 4, purified forms of LNF III inhibit the interaction of activated platelets with neutrophils and monocytes. COS cells expressing PADGEM were shown to bind to HL60 and U937 cells, whereas COS cells not expressing PADGEM did not; this interaction was inhibited by LNF III or anti-CD15 antibodies (Example 4). Thus, inhibition by LNF III

involves a process that is mediated by PADGEM on activated platelets.

Taken together, the data support a conclusion that LNF III or a portion thereof is a component of the PADGEM ligand. The LNF isomers are structurally closely related. They are composed of the same monosaccharides, but differ in the covalent linkages of these monosaccharides to form the pentassaccharide chain. LNF III binds more tightly to PADGEM, whereas LNF I demonstrates little or no interaction with PADGEM. LNF II, however, demonstrated slight inhibitory activity, particularly when the LNF to PADGEM ratio was high. Possibly minor contamination of the LNF II preparation with LNF III could account for this observation.

A comparison of the structure of LNF III, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc, to those of LNF I and LNF II (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc, Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc, respectively) indicates that the three carbohydrates share a common [GlcNAcβ1-3Galβ1-4Glc] trisaccharide moiety, but differ in the configuration of the fucosyl and galactosyl units at the non-reducing end. The preferential binding of LNF III to PADGEM suggests that LNF-III has features preferentially recognized by PADGEM. In particular, a Le^x core, comprising Galβ1-4(Fucα1-3)GlcNAc, is unique to LNF III. This suggests that α(1-3) fucosylated structures, such as α(1-3) fucosylated lactose or lactosamine are recognized by PADGEM.

The foregoing indicate that the PADGEM ligand comprises a CD15 immunoreactive carbohydrate, such as Le^x or all or portion of LNF-III. An inhibitor comprising this structure or one which mimics the CD15 positive antigen on the surface of leukocytes can interfere with PADGEM-mediated interactions.

Inhibition of PADGEM-mediated Interactions

The interaction of a PADGEM-bearing cell (e.g., a platelet, an endothelial cell) with a cell bearing a PADGEM ligand, such as a neutrophil or a monocyte, can be inhibited by contacting the PADGEM-bearing cell with an inhibitor comprising a Le^x core component. For example, Lacto-N-fucopentaose (LNF-III), a complex carbohydrate which comprises a Le^x core component and is recognized by CD15 antibodies, inhibits the binding of stimulated platelets to neutrophils (Figure 6). As shown herein, LNF III also inhibits the interaction of HL60 cells (monocyte-like cells) with COS cells that were transfected with PADGEM (Figure 7). COS cells are fibroblast-like SV40-transformed African Green Monkey kidney cells. Therefore, LNF III inhibits the adhesion involving cells which naturally express PADGEM (e.g. neutrophils and monocytes), as well as adhesion involving cells artificially induced to express PADGEM (e.g. PADGEM-transfected cells). Thus, useful inhibitors can comprise a CD15 immunoreactive carbohydrate, such as LNF III.

Identification of a 2,6-Sialic Acid Component of the PADGEM Ligand

Several studies suggest that ELAM also recognizes a Le^x core structure on the surface of leukocytes. In particular, α2,3 sialyl Le^x (SLe^x) and related structures have been suggested as the ELAM ligand (Lowe et al., Cell 63: 475-484 (1990); Phillips et al., Science 250: 1130-1132 (1990); Walz et al., Science 250: 1132-1135 (1990)). The observation that neuraminidase treatment of leukocytes greatly decreases PADGEM-mediated interaction has suggested that sialic acid may also be a part of the PADGEM ligand (Corral et al., Biochem. Biophys. Res. Comm. 172: 1349-1356 (1990)). Thus, SLe^x could be the ligand for both PADGEM and ELAM. Since PADGEM and ELAM both appear to interact with monocyte and neutrophil surface structures, the question arises of whether there are structural differences between the PADGEM and ELAM ligands, and what those differences are.

Protease digestion of HL60 cells with trypsin or proteinase K destroyed their ability to bind to activated platelets (data not shown). This result suggests that the PADGEM ligand on these cells is glycoprotein on the cell surface. Figure 8 shows the results of experiments showing that neuraminidase treatment of HL60 cells, which cleaves sialic acid residues from cell surface molecules markedly diminished the interaction of HL60 cells with CHO cells transfected with PADGEM or ELAM, consistent

with the presence of a sialic acid residue in both ligands.

However, as shown in Figure 9, results of a competition assay indicate that the dominant PADGEM and ELAM ligands, while sharing some features are distinguishable. Under the conditions used, purified PADGEM almost completely interfered with the ability of HL60 cells to bind to CHO-PADGEM transfectants. In contrast, purified PADGEM only partially inhibited the adhesion of HL60 cells to CHO-ELAM transfectants.

The PADGEM and ELAM ligands, both characterized by a terminal sialic acid and a branched trisaccharide structure consisting of Galβ1-4[Fucα1-3]GlcNAc (Le^x), differ in the linkage of the sialic acid. Figure 10 shows the results of an adhesion assay in which a highly specific lectin, which requires a disaccharide of the structure Neu5Acα2-6Gal or Neu5Acα2-6GalNAc, is shown to inhibit PADGEM-leukocyte interaction. Under the same conditions, the specific lectin did not significantly decrease ELAM-leukocyte adhesion.

The data suggest that the preferred PADGEM ligands on neutrophils and monocytes are components of a protein, characterized by a Le^x core component, and a terminal sialic acid linked α2,6 to a galactose. The 2,6-linked sialic acid residue could be linked to the galactose of the Le^x core. This structure is distinct from sialyl 2,3 Le^x previously identified as the ELAM ligand.

Inhibition of PADGEM-mediated Interactions

An inhibitor comprising all or a portion of a natural PADGEM ligand or one which mimics features of the deduced structure of the PADGEM ligand can inhibit the interaction of a PADGEM-bearing cell with a second cell bearing a PADGEM ligand. For example, the interaction of a platelet or endothelial cell with a cell, such as a monocyte or neutrophil can be inhibited by contacting the platelet or endothelial cell with an inhibitor comprising a 2,6 sialic acid component. It will be appreciated, that PADGEM need not be associated with a cell (e.g., present at the cell surface as a transmembrane protein) for inhibition of the interaction with its target ligand to occur. The interaction may be inhibited by contacting the molecule with an inhibitor. For example, a cDNA encoding a form of PADGEM which lacks the transmembrane region has been isolated from a human umbilical vein endothelial cell cDNA library (Johnston, et al., Cell 56: 1033-1044) and soluble forms of PADGEM can be constructed using recombinant techniques. The interaction of such truncated versions of PADGEM with a PADGEM-ligand can also be inhibited, reduced or prevented using an inhibitor comprising a 2,6 linked sialic acid component. This method can be useful in counteracting the effect of soluble forms of PADGEM.

Inhibitors of Leukocyte Adhesion

Inhibitors useful in the present method can be identified by their ability to inhibit (reduce or prevent) the interaction of PADGEM with its target

ligand. For inhibition or in assays of inhibition, PADGEM can be in several forms, including, but not limited to, a soluble form, incorporated into a vesicle, such as a liposome or phospholipid vesicle, or associated with a cell (e.g., as a transmembrane protein). The ligand can also be in several forms, including, but not limited to, a soluble form or associated with a cell (e.g., attached to a cell surface structure such as a glycoprotein or glycolipid). Similarly, inhibitors of the interaction of PADGEM with its target ligand can also be in several forms.

The inhibition of PADGEM-mediated binding by NeuAcα2,6 Gal-specific lectin suggests that sialic acid in the natural PADGEM ligand is terminally located, at the non-reducing end of a saccharide having two or more monosaccharide units. Thus, inhibitors useful in the present method can comprise a terminally located sialic acid, in which the sialic acid is at the non-reducing end of a saccharide having two or more monosaccharide units. Note that an internally located 2,6-linked sialic acid residue (e.g., X-NeuAc2,6-X, where X is at least a monosaccharide) may not be incompatible with inhibition, and inhibitors comprising an internal 2,6 linked sialic acid residue can be used also.

Inhibitors useful in the present method comprise a 2,6-linked sialic acid component or residue (i.e., N-acetyl neuraminic acid, NeuAc, or NANA), especially an α 2-6 linked sialic acid residue. In a second embodiment inhibitors useful in the present method

can further comprise a galactosyl residue (e.g., galactose, N-acetylgalactose) linked to sialic acid alone or in addition to a Le^x core component. For example, the inhibitor can comprise a sialyl α2,6 galactosyl component, in which a sialic acid residue linked to a galactose residue at the C-6 position of the galactose. In a second aspect of this embodiment, the inhibitor comprises a sialyl α2,6 galactosyl component in addition to a Le^x core component. In this case, a sialyl α2,6 galactosyl component and a Le^x core component can be part of a single oligosaccharide chain or on separate chains. In a third embodiment, inhibitors of the present invention can comprise a 2,6-linked sialic acid component and a Le^x core. For example, an inhibitor can comprise a 2,6-linked sialic acid component and a Le^x core in a contiguous sequence, such as α2,6 sialyl Le^x (e.g., a branched tetrasaccharide NeuAcα2,6Galβ1-4(Fucα1-3)GlcNAc), or in a non-contiguous sequence within a single saccharide chain or on separate chains (e.g., as in a glycoprotein).

As used herein, a Le^x core component refers to a structure comprising a Le^x antigen (e.g., a trisaccharide Galβ1-4(Fucα1-3)NAcGlc, an α(1-3)fucosylated lactosamine) or other α(1-3)fucosylated lactosamines exhibiting similar biological function. In addition, a Le^x core refers to a structure comprising a structural analog of a Le^x antigen, which, alone or as a component of an inhibitor, can inhibit PADGEM-mediated interactions.

The inhibitors can comprise a Le^x core or a larger carbohydrate comprising a Le^x core component. Additional sugars and/or functional groups can be added to the Le^x core consistent with inhibitory activity. For example, useful inhibitors can comprise a CD15 immunoreactive carbohydrate comprising a Le^x core, although the inhibitor itself need not be CD15 immunoreactive. Examples of CD15 immunoreactive carbohydrates comprising a Le^x core are LNF III, or a portion of LNF III comprising a Le^x core, and a Le^x antigen (e.g., Galβ1-4(Fucα1-3)GlcNAc).

CD15 antigen is a component of glycolipids, glycoprotein O-linked oligosaccharides, and glycoprotein N-linked oligosaccharides on human granulocytes. Further, as shown here, a PADGEM ligand is protease sensitive. Possibly, additional carbohydrate, protein or lipid structures of the actual ligand or ligands can contribute to the interaction with surface molecule such as PADGEM, and enhance the specificity of the interaction. Thus, useful inhibitors can comprise, for example, a protein or peptide, with a carbohydrate moiety comprising a 2,6-linked sialic acid component or other embodiment described above (e.g., a glycoprotein with N-linked and/or O-linked oligosaccharide(s)). For example, NeuAcα2,6Galβ1-4(Fucα1-3)GlcNAc- can be linked to a protein via the GlcNAc (N-acetylglucosamine) moiety or incorporated into a larger saccharide chain on a protein. Alternatively, inhibitors can comprise a

lipid portion (e.g., a phospholipid, ceramide, or sphingolipid), such as NeuAcα2,6Galβ1-ceramide. Inhibitors comprising more than one Le^x core or 2,6-linked sialic acid component may have enhanced activity due to multivalency. Inhibitors useful in the method (e.g., glycoproteins, glycolipids, carbohydrates) can also be incorporated into a lipid vesicle (e.g., phospholipid vesicle or liposome).

Inhibitors can be purified from natural sources. For example, sialylated fucosyl lactosaminoglycans can be isolated from granulocytes (Fukuda, et al., J. Biol. Chem. 259: 10,925-10,935 (1984)). Alternatively, they can be synthesized chemically or enzymatically using techniques known in the art (Toone, E. et al., Tetrahedron Rep., 45: 5365-5422 (1989); Wong, C.-H., Science, 244: 1145-1152 (1989)).

The activity of an inhibitor may be monitored using an appropriate assay. For example, the adhesion assays described in Example 2 can be used to assay the inhibitory activity of candidates upon PADGEM-mediated adhesion. Alternatively, a candidate inhibitor may be identified by its ability to interfere with the interaction between an identified inhibitor (e.g., LNF III) and PADGEM (e.g., purified PADGEM, PADGEM on a cell, PADGEM in a liposome) in a competitive binding assay.

Methods of Therapy

PADGEM on the surface of platelets is thought to be have an important role in the clotting process. Moreover, PADGEM, which is also present in

endothelial cells is thought to be involved in the recruitment of neutrophils and monocytes to sites of inflammation. By inhibiting (reducing or preventing) the interaction or adhesion of endothelial cells or platelets with white blood cells (i.e., leukocytes, such as monocytes and neutrophils) by the method of the present invention, it is possible to interfere with the processes that these cell interactions mediate or participate in.

For example, in one embodiment, the inhibition of the interaction of activated platelets with neutrophils and monocytes is inhibited by contacting the platelets with an inhibitor comprising a 2,6 linked sialic acid component. Activated platelets can bind to injured endothelial and subendothelial surfaces through mechanisms involving glycoprotein Ib and von Willebrand factor. The expression of PADGEM on these platelets at the site of vascular injury could lead to the binding of monocytes and neutrophils. The latter cells are capable of initiating the tissue factor-mediated extrinsic pathway of blood coagulation. However, the inhibitors of this invention can interfere with platelet-neutrophil or platelet-monocyte interactions to block adhesion and thereby interrupt the coagulation process. Thus, it is possible to inhibit pathological thrombosis using the present method.

In another embodiment, activated platelets or endothelial cells at the site of tissue injury or inflammation could recruit leukocytes from the blood stream, resulting in the release of inflammatory

mediators and causing further tissue damage. For example, where PADGEM-mediated interactions have a role in inflammation, an inhibitor comprising a 2,6 linked sialic acid component can inhibit the adhesion of monocytes and neutrophils to platelets or endothelial cells, to prevent or minimize inflammation. Thus, autoimmune and inflammatory diseases or conditions can be treated by the present method.

Tissue injury, such as neutrophil-mediated ischemia-reperfusion damage due to blood vessel occlusion and reperfusion could be inhibited by interfering with adhesion of neutrophils. Contacting platelets bearing PADGEM with an inhibitor comprising a 2,6 linked sialic acid component and/or a Le^x core can inhibit neutrophil adhesion, minimizing damage in the region of the thrombus. Treatment with clot-dissolving drug, such as tissue plasminogen activator or streptokinase, can be accompanied by treatment with an inhibitor comprising a Le^x core to inhibit reperfusion injury. At the same time, the inhibitor can also act together with clot-dissolving drugs to inhibit clotting.

In a model of atherosclerosis, injured endothelial cells in a vessel wall express PADGEM on their surface. Monocytes bearing a PADGEM ligand are recruited to the site by virtue of PADGEM-PADGEM ligand interaction, and adhere to the endothelial cells. The monocytes become pathological foam cells by ingestion of lipids, platelet fragments, and other molecules. However, the atherosclerotic process can be inhibited by contacting the PADGEM-bearing

endothelial cells with an inhibitor of the present invention, which inhibits PADGEM-mediated adhesion.

CD15 antigen, comprising a Le^x core, is a marker for adenocarcinoma of the lung, colon and stomach, and for certain forms of lymphoma (Hall and Ardenne, J. Clin. Pathol., 40: 1298-1304 (1987); Sanders et al., J. Pathol., 154: 255-266 (1988)). It is possible that cell adhesion processes play a role in metastasis. For example, ELAM-1 supports the adhesion of a human colon carcinoma cell line to endothelial cells (Rice and Bevilacqua, Science, 246: 1303-1306 (1989)). Adhesion to vessel walls and estravasation by certain tumor types may be facilitated by their expression of a ligand for PADGEM. It is possible to disrupt the metastatic process by inhibiting the interaction of PADGEM with complementary tumor cell antigens by the method of the present invention.

For use in treating a condition in an individual in which a surface molecule capable of interacting with a Le^x core plays a role in pathological process (e.g., atherosclerosis, thrombosis, inflammation, or metastasis), inhibitors of the present invention are administered by an appropriate route (e.g., intravenously, parenterally or topically). Treatment is under appropriate conditions and in amounts sufficient to reduce or prevent adhesion and thereby, reduce or prevent the disease process. For example, an inhibitor can be combined with a suitable carrier, incorporated into a liposome, or polymer release system for administration.

The invention is further and more specifically described in the following examples.

EXAMPLES

The reagents and cell preparation procedures below were used in the following examples.

Reagents

Antibody 80H5 was purchased from AMAC, Inc. Other antibodies were the generous gifts of Drs. Dennis Hickstein and John Harlan (7C3), Dr. Paul Guyre (PM81, 168, AML-2-23), and Dr. Douglas Faller (TS1/18, OKM15, TS2/9, W6/32, LB3.1, GAP8.3, 4F2, and 63D3). Polyclonal anti-PADGEM antibodies were raised in rabbits and isolated by affinity chromatography on PADGEM-Sepharose, as previously described (Berman et al., J. Clin. Invest., 78: 130-137 (1986)). The monoclonal anti-PADGEM antibody AC1.2 has been previously described (Larsen et al., Cell 59: 305-312 (1989)). LNF I, LNF II, and LNF III, purchased from Calbiochem, were greater than 95% pure by HPLC, as assayed by the supplier.

Isolation of Cells

Platelets were isolated by gel filtration from fresh anticoagulated blood obtained from normal human donors (Hsu-Lin et al., J. Biol. Chem., 259: 9121-9126 (1984)). Activated platelets were prepared by incubating cells without stirring for 20 minutes

at 22° C with thrombin at a final concentration of 0.25 U/ml. Fresh platelets were used in cell adhesion assays within 30 minutes of preparation.

Neutrophils were prepared by the method of English and Anderson (J. Immunol. Method, 5: 249-252 (1974)). The neutrophil preparations were greater than 95% pure by light microscopy. Monocytes were prepared by washing the mono-nuclear leukocyte fraction twice with human serum-5mM EDTA and incubating the cells in RPMI 1640-10% fetal calf serum in sterile plasmid dishes for 2 hours at 37° C. The dishes were washed three times with PBS at 37° C to remove nonadherent cells. PBS at 0° C was added, and the cells were incubated at 4° C for 1 hour. Adherent cells were gently detached with a rubber policeman, washed in PBS, and resuspended in RPMI 1640-1% fetal calf serum. Lymphocytes were obtained by washing the nonadherent cells with PBS and resuspending these cells in RPMI 1640-1% fetal calf serum. The purity of these preparations was established to be greater than 90% by light microscopy using Wright esterase and nonspecific esterase stains.

Cell lines HL60 and U937 were maintained in culture in RPMI 1640 medium supplemented with penicillin G sodium (100 U/ml), streptomycin sulfate (100 μg/ml), HEPS (10 mM), sodium pyruvate (1 mM), L-glutamine (2 mM), β-mercaptoethanol (0.0004%), and 10% fetal calf serum.

Example 1

Cloning of PADGEM and Expression in COS Cells

The PADGEM cDNA was cloned from a human umbilical vein cDNA library in λgt11 using oligonucleotides based upon the published DNA sequence (Johnston et al., Cell 56: 1033-1044 (1989)). Approximately 3 x 10^6 plaques from an oligo(dT)-primed human umbilical vein endothelial cell cDNA library were transferred to nitrocellulose filters for screening. Duplicate filters were hybridized with either a ^{32}P-labeled 24 nucleotide probe derived from the 5′ end of the translated sequence or one from the 3′ end of the translated sequence (Johnston et al., Cell 56: 1033-1044 (1989)). Of six clones that were positive with both probes, only one appeared to be a full-length cDNA. Sequencing demonstrated that the latter clone lacked 56 bases from the 5′ end of the translated sequence. One of the base differences from the original published sequence, a T to C change at position 99, resulted in an additional EcoRI site that may be responsible for the 56 base deletion. The partial PADGEM cDNA was rendered full length by ligating to it a synthetic DNA fragment containing the 56 bp of missing sequence.

The sequence of the full-length PADGEM cDNA was established in its entirety. The nucleotide sequence obtained was identical to that of Johnston et al. (Cell 56: 1033-1044 (1989)), with the

exception of five nucleotides within the coding sequence: T at 1088, C at 1832, C at 1850, C at 99, and C at 859. The latter two sequence differences result is amino acid sequence differences, such that a proline is encoded at residue -21 instead of serine, and a threonine is encoded at residue 233 instead of isoleucine. The other three base changes do not alter the predicted amino acid sequence. The full-length PADGEM cDNA was inserted into a modified form of the expression vector CDM8 (Tedder and Isaacs, J. Immunol., 143: 712-717 (1989)).

COS cells (1 X 10^5) were transfected with 40 μg of the resulting PADGEM expression vector by calcium phosphate precipitation. Coverslips (12 x 12 mm) were added to each culture. After 48 hours of growth in DMEM-10% fetal calf serum, the COS cells were confluent.

The presence of PADGEM expression in the transfected COS/PADGEM cells was demonstrated by immunofluorescent staining using the monoclonal antibody AC1.2. Cells were incubated with the anti-PADGEM monoclonal antibody AC1.2 (Bonfanti et al., Blood 73: 1109-1112 (1989)) and stained with rhodamine conjugated to goat anti-mouse antibody. The immunoflourescence data indicated that, in these experiments, 10%-20% of the COS cells expressed PADGEM. Furthermore, HL60 cells, from a human cell line that exhibits monocyte-like characteristics, and which bind to platelets in a PADGEM-dependent manner (Larsen, E. et al., Cell 59: 305-312 (1989)) were found to bind to COS cells expressing PADGEM

(COS/PADGEM transfectants). In contrast, the HL60 cells did not bind to COS cells that were subjected to mock transfection. These results indicated that the COS-PADGEM transfectants retain adhesive properties of PADGEM.

Example 2

Cell Adhesion Assays

Phase-contrast Assay

Twenty microliters of platelet suspension (2×10^8/ml) was mixed with 20 μl of cell suspension (2×10^6/ml) and incubated for 20 minutes at 22° C in a microfuge tube. An aliquot of the cell suspension was then in a Neubauer chamber and evaluated by light microscropy using an Olympus model BH-2 microscope. Three samples from each assay were evaluated by counting 200 cells and scoring the percentage of cells with two or more adherent platelets (Jungi et al., Blood 67: 629-636 (1986)). Antibody inhibition studies were performed by preincubating cells (20 μl; 3×10^6/ml) with 20 μl of antibody solution for 20 minutes at 22° C. Subsequently, 20 μl of platelet suspension was added, and the mixture was incubated for 20 minutes at 22° C. Samples were analyzed as above.

COS Cell-PADGEM Adhesion Assays

HL60 cells (1×10^7), maintained in culture, were washed and resuspended in 0.5 ml of serum-free

RPMI 1640. The cells were labeled with 270 μCi of ^{111}In oxine (Callow et al., Arch. Surg., 117: 1447-1455 (1982)) and 10 μg of bis-carboxyethyl-carboxyfluorescein (Kolber et al., J. Immunol. Meth., 108: 255-264 (1988)) by incubation at 37° C for 30 minutes. After washing with RPMI 1640-1% bovine serum albumin, the cells (1×10^6) were incubated with the coverslips containing confluent COS cell transfectants, in the presence or absence of LNF isomers, for 20 minutes at 37° C. The coverslips were washed with RPMI 1640, and duplicate coverslips were assayed for ^{111}In activity. Alternatively, samples were evaluated for HL60 cell adherence by fluorescence and phase-contrast microscopy using a Zeiss Axioscope microscope in a blind assay. Inhibition of the binding of COS/PADGEM cells to U937 cells by the antibody 80H5 was performed in experiments identical to the procedure described above.

Binding of U937 Cells to Phospholipid Vesicles Containing PADGEM

PADGEM was incorporated into phospholipid vesicles as previously described (Larsen et al., Cell 59: 305-312 (1989)) with some modifications. Briefly, 5 mg of egg phosphatidylcholine (Avanti Polar Lipids) and 0.025 mg of Di $IC_{16}(3)$ (1,1′-dihexadecyl-3,3,3′,3′-tetramethyl-lindocarbocyanine perchlorate) (Molecular Probes) in chloroform were mixed, and the chloroform was removed by evaporation at 37° C under nitrogen. The dried

lipids were resuspended in methylene chloride, and the solvent was removed by evaporation. Purified PADGEM (1 ml; 65 μg/ml; Larsen et al., Cell 59: 305-312 (1989)) in Tris-buffered saline containing 50 mM octyl-β-D-glucopyranoside (Calbiochem) or Tris-buffered saline along containing 50 mM octyl-β-D-glucopyranoside (1 ml) was added to the dried phospholipids, and the lipids were resuspended. The preparations were dialyzed under nitrogen against Tris-buffered saline-0.02% NaN_3 for 24 hours. Vesicles were separated from free protein by gel filtration on a Sepharose 4B column. Phospholipid vesicles (50 μl) with or without PADGEM were incubated with 2 x 10^5 U937 cells in RPMI 1640, 1% fetal calf serum, 2% bovine serum albumin for 30 minutes at 23° C. For experiments with 80H5 antibody, U937 cells were incubated with the antibody (5 μg/ml) for 1 hour; phospholipid vesicles were added, and the incubation was continued for an additional 30 minutes. Prior to analysis on a FACScan (Becton Dickinson), each sample was diluted 10 fold with RPMI 1640, 1% fetal calf serum, 2% bovine serum albumin. U937 cells were identified by their forward and side light scatter profiles, and binding of PADGEM in phospholipid vesicles was quantitated by measuring red fluorescence. Data were collected for 3000 cells.

Example 3

Anti-CD15 Antibodies Inhibit the Platelet-Leukocyte Interaction

Effects of Anti-Leukocyte Antibodies on the Interaction of Activated Platelets with Neutrophils, Monocytes, HL60 Cells, and U937 Cells

Thrombin-activated platelets bind to human neutrophils, monocytes, HL60 cells and U937 cells in an interaction that is mediated by PADGEM on the surface of the platelet (Larsen et al., Cell 59: 305-312 (1989). This interaction is inhibited by anti-PADGEM antibodies and purified PADGEM. Unstimulated platelets, which do not express PADGEM on the platelet surface, do not interact with these leukocytes.

To identify the PADGEM recognition site on leukocytes that mediates the binding of activated platelets, monoclonal antibodies directed at various antigens on the surface of monocytes and neutrophils were tested for their ability to inhibit the interaction of these cells with activated platelets, using the phase-contrast cell adhesion assay described in Example 2 (Figure 1 and Figure 2). The antibodies were raised against various leukocytes and myeloid cell lines and are directed at leukocyte antigens. The antibodies which were tested and their corresponding antigens are as follows: TS1/18, LFA-1 (β); OKM15, CR3; TS2/9, LFA-3; W6/32, HLA class I;

LB3.1, HLA class II; GAP8.3, T200; 4F2, 4F2; 63D3, 63D3; 168, 168; AML-2-23, 2-23; PM81, CD15; 7C3, CD15; 80H5, CD15. These immunochemical reagents included antibodies of the IgG and IgM isotype. The effect of buffer alone (HEPES) on the adherence of activated platelets and neutrophils served as a negative control, while the effect of anti-PADGEM antibodies on cell adherence served as a positive control for inhibition. The percentage of cells displaying two or more adherent platelets was determined as described in Example 2.

With the exception of antibodies which recognize CD15 (PM81, 7C3, and 80H5), none of the other antibodies that were tested demonstrated inhibitory properties. The anti-CD15 monoclonal antibodies, obtained from three separate and independent hybridoma cell lines and of the IgM isotype, each displayed significant inhibition of the interaction between neutrophils and activated platelets (Figure 1). These results suggest that the anti-CD15 antibodies are targeted against a structure on the leukocyte surface which participates in the PADGEM-mediated binding of leukocytes to activated platelets.

The effect of 80H5 antibodies against CD15 on the interaction of activated platelets with neutrophils, HL60 cells, U937 cells, or monocytes is illustrated in Figure 2. In the absence of anti-CD15 antibodies (black bars), activated platelets adhered to neutrophils (PMN). However, this binding was inhibited with anti-CD15 antibodies. Similarly, the

80H5 antibody blocked the interaction of activated platelets with monocytes (Mono), U937 cells, and HL60 cells. These leukocytes are known to be CD15 positive, and anti-CD15 antibody was observed to inhibit cell adhesion with thrombin stimulated platelets in each case. In contrast, we confirmed that platelets, which carry PADGEM, but which apparently lack the PADGEM ligand, are CD15 negative. Thus, the distribution of CD15 positivity parallels the expression of PADGEM recognition sites on specific leukocytes (Larsen et al., Cell 59: 305-312 (1989)). Just as anti-PADGEM antibodies directed against PADGEM on platelets can inhibit platelet-leukocyte interaction, anti-CD15 antibodies directed against CD15 on leukocytes inhibit platelet-leukocyte interaction.

Inhibition of PADGEM-Leukocyte Binding with CD15 Antibodies

The inhibition of activated platelet adherence to neutrophils by anti-CD15 antibody was dependent upon the concentration of antibody. Using the anti-CD15 antibody 7C3 (Nauseef et al., Blood 62: 636-644 (1983)) in the phase-contrast adhesion assay (Example 2), half-maximal inhibition was observed at about 30 μg/ml (Figure 3). Although complete inhibition was not observed, inhibition to the extent of 60%-80% was observed in multiple, independent experiments. Similar results were obtained with other anti-CD15 antibodies, including PM81 and 80H5, or with different cells, including monocytes, HL60

cells, and U937 cells (data not shown). It has been previously demonstrated that the binding of leukocytes (including neutrophils, monocytes, HL60 cells, and U397 cells) to activated platelets is mediated by PADGEM (Larsen, E. et al., Cell 59: 305-312 (1989)). The results shown here suggest that antibodies to CD15, which disrupt cell-cell interactions which are mediated by PADGEM, are directed toward the PADGEM ligand.

To confirm that the inhibitory activity of the anti-CD15 antibodies involves the PADGEM ligand specifically, the effect of anti-CD15 antibodies on the binding of COS/PADGEM cells to ^{111}In-labeled U937 cells was studied. COS/PADGEM cells were constructed as described in Example 1. The COS cell-PADGEM adhesion assay is described in Example 2. As shown in Figure 4, anti-CD15 antibody 80H5 inhibited COS/PADGEM binding to U937 cells, indicating that the anti-CD15 antibodies specifically interfere with PADGEM-mediated interactions. These results further emphasize that the anti-CD15 antibodies are directed against the PADGEM ligand, and not a ligand of other proteins that have been implicated in platelet-leukocyte interaction (Silverstein and Nachman, J. Clin. Invest., 79: 867-874 (1987)).

To demonstrate further that the anti-CD15 antibody inhibition of leukocyte-platelet interaction was mediated via PADGEM, the effect of antibodies against CD15 on the binding of PADGEM-containing phospholipid vesicles to U937 cells was determined. Purified PADGEM was incorporated into fluorescently

labelled phospholipid vesicles and adhesion of vesicles to U937 cells was monitored on a fluorescence-activated flow cytometer as described in Example 2. As shown in Figure 5, anti-CD15 antibodies inhibited the interaction of U937 cells with phospholipid vesicles containing PADGEM. Phospholipid vesicles lacking PADGEM did not interact with U937 cells, confirming previous results (Larsen et al., Cell 59: 305-312 (1989)). These results indicate that PADGEM is the complementary structure that is recognized by the target of the anti-CD15 antibody.

Example 4

Inhibition of PADGEM-Mediated Platelet-Leukocyte Interaction by Lacto-N-Fucopentaose III

CD15 antigen has been identified as a complex carbohydrate; CD15 antibodies react with lacto-N-fucopentaose III (LNF III). This carbohydrate has the structure Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc. If the anti-CD15 antibody inhibits the interaction of stimulated platelets and leukocytes by binding the PADGEM recognition site of leukocytes, thus precluding the binding of PADGEM on platelets, purified CD15 antigen (e.g., LNF III) should also inhibit platelet-leukocyte interaction, since it would saturate the binding sites on PADGEM.

As shown in Figure 6, LNF III was an effective inhibitor of the adherence of activated platelets to neutrophils, as determined using the direct cell adhesion assay (closed squares). Half-maximal inhibition was observed at about 50 μg/ml. Two LNF III isomers, known as LNF I (Fucα1-2Galβ1-3NAcGlcβ1-4Glc) and LNF II (Galβ1-3(Fucα1-4)NAcGlcβ1-3Galβ1-4Glc), were also tested for inhibitory activity. The three LNF isomers are structurally closely related. They are composed of the same monosaccharides, but differ in the covalent linkages of these monosaccharides to form the pentassaccharide chain. Under the conditions of these experiments, neither LNF I (open squares) nor LNF II (closed circles) had inhibitory activity on cell adhesion (Figure 6).

Although LNF III inhibited the interaction of activated platets and neutrophils, LNF III did not alter cell viability, as determined using the trypan blue exclusion method. In addition, a similar inhibitory effect of LNF III on platelet-HL60 cell and platelet-U937 cell interactions was demonstrated (data not shown).

To address the possibility of a nonspecific effect of LNF III on cell-cell interaction involving platelets, the effect of LNF III on ADP-induced platelet aggregation was examined. Platelet aggregation, which involves the binding of fibrinogen to glycoprotein IIb-IIIa, was equivalent in the presence or absence of LNF III. This example, in which cellular adhesion dependent upon glycoprotein

IIb-IIIa was not affected by LNF III, suggests that LNF III specifically interferes PADGEM-mediated cell adhesion.

To extend this result, the effect of different concentration of LNF isomers on the binding of COS/PADGEM cells to HL60 cells was also studied. The results of a COS cell-PADGEM adhesion assay, in which the binding of ^{111}In and bis-carboxyethyl-carboxyfluorescein-lableled HL60 cells to COS/PADGEM cells was monitored by assaying ^{111}In activity (Example 2), are shown in Figure 7. LNF III significantly inhibited the binding of radiolabeled HL60 cells to COS/PADGEM cells (closed square). In contrast, LNF I (open squares) did not inhibit this interaction and LNF II (closed circles) had only a small inhibitory effect. The inhibitory effect of LNF II was more apparent when the LNF to PADGEM ratio was high. These results suggest that PADGEM binds LNF III preferentially but may have some affinity for LNF II. It is possible that minor contamination of the LNF II preparation with LNF III can account for the inhibitory activity observed.

The inhibitory effects of LNF I, II, and III on the interaction of HL60 cells and COS/PADGEM was also studied morphologically in a blind assay. In these experiments, the binding of fluorescently labeled HL60 cells to COS/PADGEM cells was scored (Example 2). LNF III demonstrated significant inhibitory activity. In contrast, LNF I and LNF II demonstrated no inhibitory activity, and cell adhesion was comparable to assays in which LNF was absent. Mock-transfected COS cells did not bind HL60 cells.

The data demonstrate that LNF III specifically interferes with PADGEM-mediated cell-cell interactions. The inhibition of PADGEM-mediated cell-cell interactions by anti-CD15 monoclonal antibodies and CD15 antigen (e.g., LNF III), suggests that the PADGEM ligand on leukocytes shares structural features with CD15 positive cell surface structures (CD15 antigens), such as LNF III or Le^x, or a portion thereof.

Example 5

Effect of Neuraminidase and Proteases on HL60 Cell Interaction with PADGEM

To determine whether the PADGEM ligand is associated with protein, HL60 cells were treated with trypsin or proteinase K and then tested for their ability to bind activated platelets. HL60 cells were incubated with trypsin or proteinase K for 5-120 minutes at 24°C. Protease digestion of HL60 cells destroyed the ability of activated platelets to bind to HL60 cells (data not shown). These results suggest that the complete PADGEM ligand is located on a glycoprotein and not on glycolipid associated with the cell surface.

As shown in Figure 8, incubation of HL60 cells with vibrio cholerae neuraminidase, arthrobacter ureafaciens neuramininidase or Newcastle disease virus neuraminidase significantly decreased the interaction of HL60 cells with both CHO-PADGEM

(hatched bar) and CHO-ELAM (solid bar) as compared with untreated CHO-PADGEM or CHO-ELAM control cells. The adhesion assay used was similar to the COS-PADGEM adhesion assay using ^{111}In-labelled HL60 cells. CHO-PADGEM and CHO-ELAM cells were constructed by transfecting CHO-DUKX cells with a cDNA encoding either PADGEM or ELAM-1.

Although Vibrio cholerae and Arthrobacter ureafaciens neuraminidases are of broad specificity and Newcastle disease virus neuraminidase is thought to be specific for α2,3- or α2,8-linked sialic acid, the possibility of non-specific cleavage or contaminating activities makes these results difficult to interpret. However, these results are consistent with the results of Corral et al., in indicating that the removal of sialic acid from HL60 cells markedly diminished PADGEM-mediated cell interaction (Biochem. Biophys. Res. Commun., 172: 1349-1356 (1990)). The data confirm the contribution of sialic acid to the PADGEM-PADGEM ligand interaction.

Example 6

PADGEM and ELAM-1 Bind to Distinct But Overlapping Ligands on Leukocytes

A competition assay was employed to determine whether purified PADGEM can inhibit the interaction of CHO-ELAM with HL60 cells, as it does with activated platelets. The results of this assay are

shown in Figure 9. The data indicate that purified PADGEM inhibits the interaction of HL60 cells with CHO-PADGEM (open circles). Under the conditions employed, only partial inhibition of CHO-ELAM binding to HL60 cells was observed in the presence of purified PADGEM (closed circles). While half-maximal inhibition of binding of CHO-PADGEM cells to HL60 cells was observed at 2 μg/ml of PADGEM, approximately 50-fold greater concentrations of PADGEM were necessary to effect comparable inhibition of CHO-ELAM binding to HL60 cells. These results demonstrate that the dominant PADGEM ligand or ligands are distinct from the ELAM ligand on HL60 cells. Based upon the observation that sialyl 2,3 Le^x is the ELAM ligand, these results suggest that PADGEM may interact with sialyl 2,3 Le^x or that PADGEM sterically interferes with the ELAM-ELAM ligand interaction. However, as shown below, the dominant PADGEM ligand does not appear to be sialyl 2,3 Le^x.

Example 7

NANA 2,6-specific Lectin Inhibits PADGEM-leukocyte Interaction

Lectin Inhibition Assay

Each of the following cell types, Chinese hamster ovary cells-DUKX (CHO-DUKX), Chinese hamster ovary cells transfected with cDNA for ELAM (CHO-ELAM) and Chinese hamster ovary cells transfected with cDNA

for PADGEM (CHO-PADGEM), were separately plated at a density of 6 x 10^4 cells per milliliter of medium into Costar 48 well culture plates. On the following day, HL60 cells in culture were sedimented by centrifugation and resuspended in medium (RPMI, 10% fetal calf serum) to a density of 5 x 10^5 cells/ml. Tritiated thymidine (5 μCi per milliliter) was added to the HL60 cell suspension and the cells were grown overnight. The following day the HL60 cells were washed trice in serum free RPMI and resuspended in a volume of 5 mls of RPMI. After assuring that the free tritium in the cell suspension is no more than 10% of the cell associated tritium, the cell count of the cell suspension was established and the cell density was adjusted to 1 x 10^6 cells per milliliter.

To perform the assay medium was aspirated from the CHO cells, CHO-ELAM cells and CHO-PADGEM cells. The wells containing these cells were rinsed with 1 ml of serum free RPMI. HL60 cells were preincubated for 10 minutes with the lectin Sambucus nigra (E-Y Laboratories, San Mateo, CA, catalogue number BA6801) at the concentrations indicated. Aliquots of these HL60 cells (100,000 to 300,000 cells) were then added to individual wells containing CHO cells of the various types and the two cell populations incubated together for 30 minutes at room temperature. Unbound HL60 cells were removed by aspiration and the wells were washed trice with serum free RPMI. The adherent CHO cells and any bound HL60 cells were detached from the surface of the wells with 250 μl of phosphate buffered saline containing 1 mM EDTA. An aliquot of

the detached cells (200 μl) was analyzed for tritium content in a β-scintillation counter. The level of nonspecific binding of tritiated HL60 cells was taken as the level of binding seen in the wells containing the CHO-DUKX cells (parent cell line of CHO-PADGEM and CHO-ELAM transfectants). This value was subtracted from the level of tritiated HL60 cells bound in wells containing CHO-ELAM or CHO-PADGEM cells. The decrease in binding induced by the presence of the lectin was determined by comparing the level of binding of HL60 cells in the presence of the lectin to that observed in its absence.

NANA 2,6-specific Lectin Inhibits PADGEM-leukocyte Interaction

To investigate whether different isomers of sialyl Le^x (SLe^x) represent the PADGEM and ELAM ligands, a highly specific lectin was used to determine if it would specifically inhibit cell adhesion (Knibbs, R.N. et al., J. Biol. Chem., 266: 83-88 (1991)). Sambucus nigra lectin, which requires a disaccharide Neu5Acα2-6Gal or Neu5Acα2-6GalNAc for binding, inhibited the interaction of CHO-PADGEM with HL60 cells; half-maximal inhibition under the conditions employed was observed at 1-2 μg/ml (Figure 10). This lectin exhibited minimal inhibitory effect on CHO-ELAM binding to HL60 cells. Furthermore, CHO cells expressing neither PADGEM nor ELAM failed to bind HL60 cells in the presence or absence of Sambucus nigra lectin (not shown). These results are consistent with the interpretation that sialyl α2,6

Gal is an important component of the PADGEM ligand, in contrast to the ELAM ligand.

A number of sugars were tested for inhibition of PADGEM- and ELAM-mediated interactions using the same lectin inhibition assay, substituting specific sugars for the lectin. Each of the following sugars were tested at a single concentration:

3′-sialyllactose (i.e., NeuAcα2-3Galβ1-4Glc);

6′-sialyllactose (i.e., NeuAcα2-6Galβ1-4Glc);

3-fucosyllactose (i.e., Galβ1-4[Fucα1-3]Glc);

3′-sialyl-3-fucosyllactose
(i.e., NeuAcα2-3Galβ1-4[Fucα1-3]Glc).

3′-sialyllactose and 6′-sialyllactose were incubated with cells at a concentration of 0.5 mM, and 3-fucosyllactose and 3′sialyl-3-fucosyllactose were incubated with cells at a concentration of 0.25 mM. Under the conditions used, none of the four sugars tested appreciably inhibited PADGEM-mediated binding of HL60 cells to CHO-PADGEM transfectants. However, NeuAcα2-3Galβ1-4[Fucα1-3]Glc decreased ELAM-mediated binding by 50-60%. The other three sugars behaved similarly in inhibition of ELAM-mediated binding, inhibiting adhesion by no more than 10% in any case under the conditions of the assay. Note that these experiments were done once only and at a single concentration in each case. It is possible that

different conditions or higher concentrations may be needed to observe inhibition of PADGEM-mediated binding. Also, 3′-sialyllactose and 6′-sialyllactose lack an α1,3 fucosyl residue typical of Le^x core components.

CLAIMS

1. A method of inhibiting the adhesion of a first cell bearing PADGEM with a second cell bearing a PADGEM ligand comprising contacting the first cell with an inhibitor comprising a 2,6-linked sialic acid component under conditions whereby adhesion is inhibited.

2. The method of Claim 1, wherein the 2,6-linked sialic acid component is terminally located.

3. The method of Claim 1, wherein the inhibitor further comprises a galactosyl residue selected from the group consisting of: galactose and N-acetyl galactose, and the sialic acid component is on the non-reducing end and is attached to the galactosyl residue via an $\alpha 2,6$ linkage.

4. The method of Claim 1 wherein the inhibitor further comprises a Le^x core.

5. The method of Claim 4 wherein the Le^x core component is CD15 positive.

6. The method of Claim 5 wherein the CD15 positive Le^x core component is selected from the group consisting of: CD15 antigen, LNF-III and Le^x.

7. The method of Claim 4 wherein the inhibitor comprises α2,6-linked sialyl Le^x.

8. The method of Claim 1 wherein the first cell is a platelet.

9. The method of Claim 8 wherein the second cell is a leukocyte selected from the group consisting of: monocytes and neutrophils.

10. The method of claim 1 wherein the first cell is an endothelial cell.

11. The method of claim 10 wherein the second cell is a leukocyte selected from the group consisting of: monocytes and neutrophils.

12. The method of Claim 1 wherein the inhibitor comprising a Le^x core is selected from the group consisting of: a glycoprotein, a carbohydrate and a glycolipid.

13. A method of inhibiting the adhesion of PADGEM with a PADGEM ligand comprising contacting PADGEM with an inhibitor comprising a terminal 2,6-linked sialic acid.

14. A method of treating an individual to reduce adhesion of leukocytes to platelets or endothelial cells comprising administering an inhibitor comprising a 2,6-linked sialic acid residue in a therapeutically effective amount.

15. An inhibitor of PADGEM-mediated cell-cell interaction comprising a 2,6-linked sialic acid component for use as a medicament.

16. Use of an inhibitor of PADGEM-mediated cell-cell interaction, the inhibitor comprising a 2,6-linked sialic acid component, for the manufacture of a medicament for treatment of a condition in which a cell surface molecule capable of interacting with a Le^x core plays a role in a pathological process, e.g., atherosclerosis, thrombosis, inflammation, ischemia-reperfusion or metastasis.

17. An inhibitor of PADGEM-mediated cell-cell interaction comprising a 2,6-linked sialic acid component.

18 An inhibitor of claim 17 further comprising a galactosyl residue.

19. An inhibitor of Claim 17 further comprising a CD15 immunoreactive component.

20. An inhibitor of Claim 17 further comprising a Le^x core component.

21. An inhibitor of Claim 17 wherein the 2,6-linked sialic acid component is an α 2,6-linked sialic acid residue.

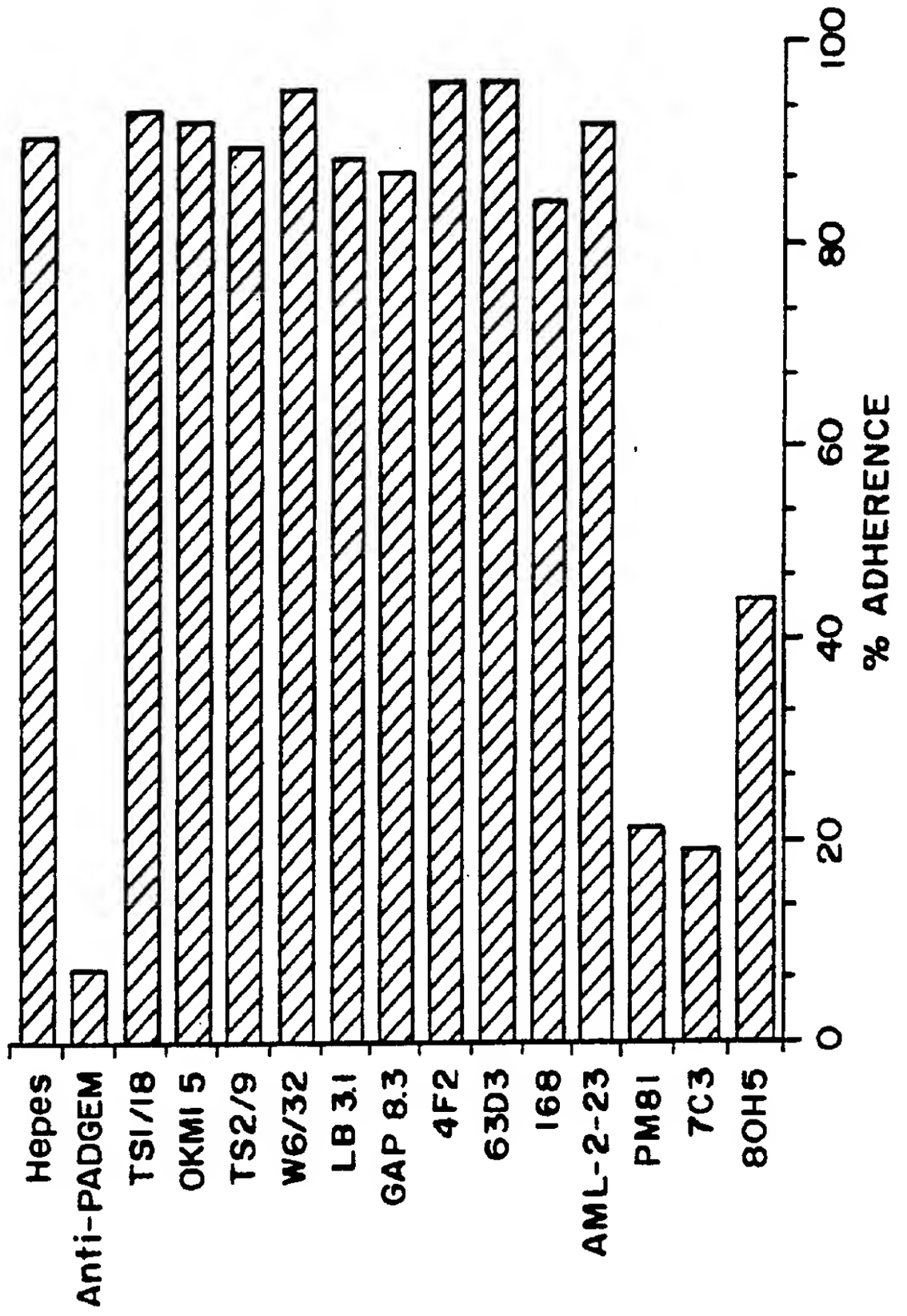
Hepes
-PADGEM
TSI/18
OKMI 5
TS2/9
W6/32
LB 3.1
GAP 8.3
4F2
63D3
168
ML-2-23
PM81
7C3
8OH5
Anti
A
0
20
40
60
80
100
% ADHERENCE

FIG. 1

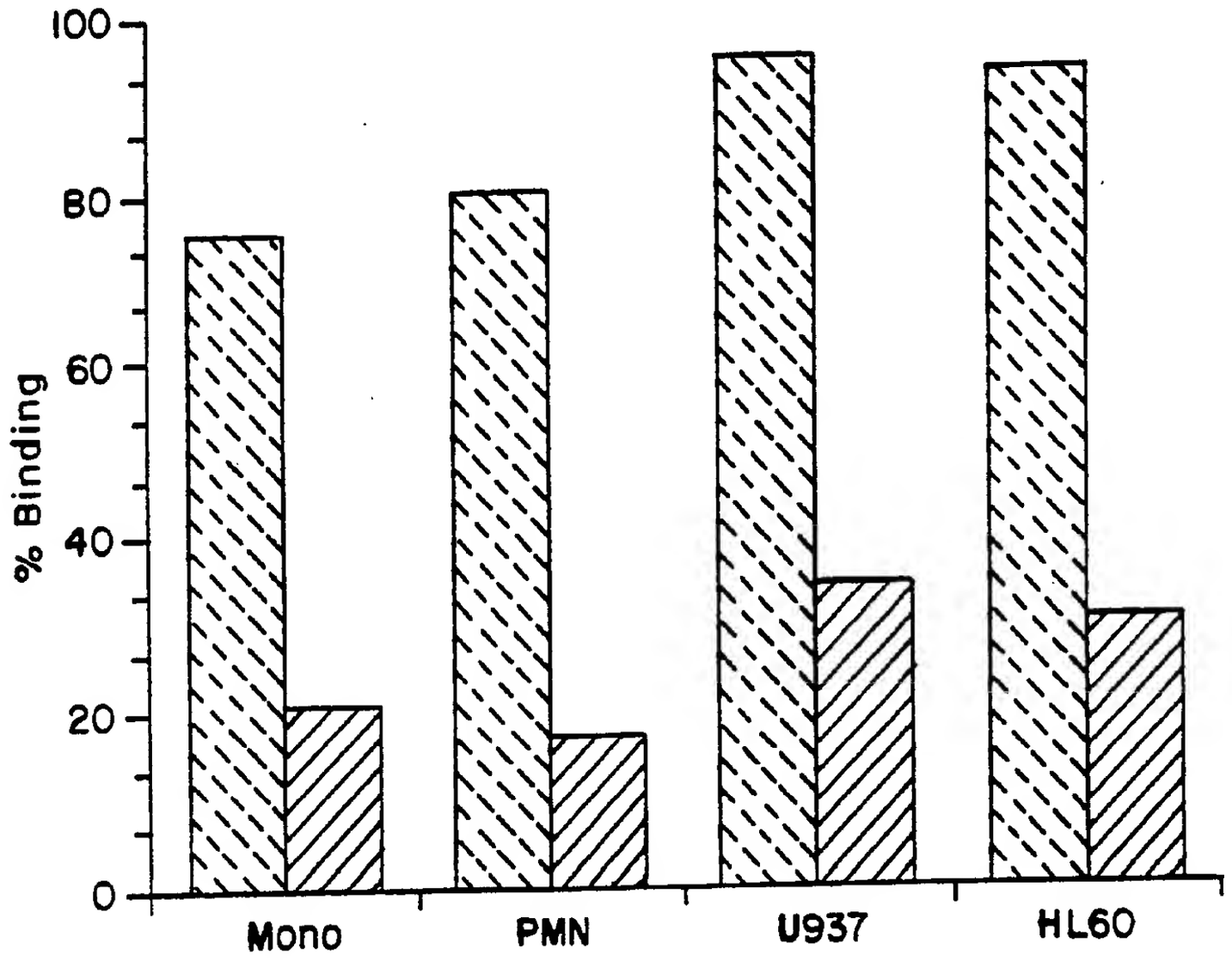

FIG. 2

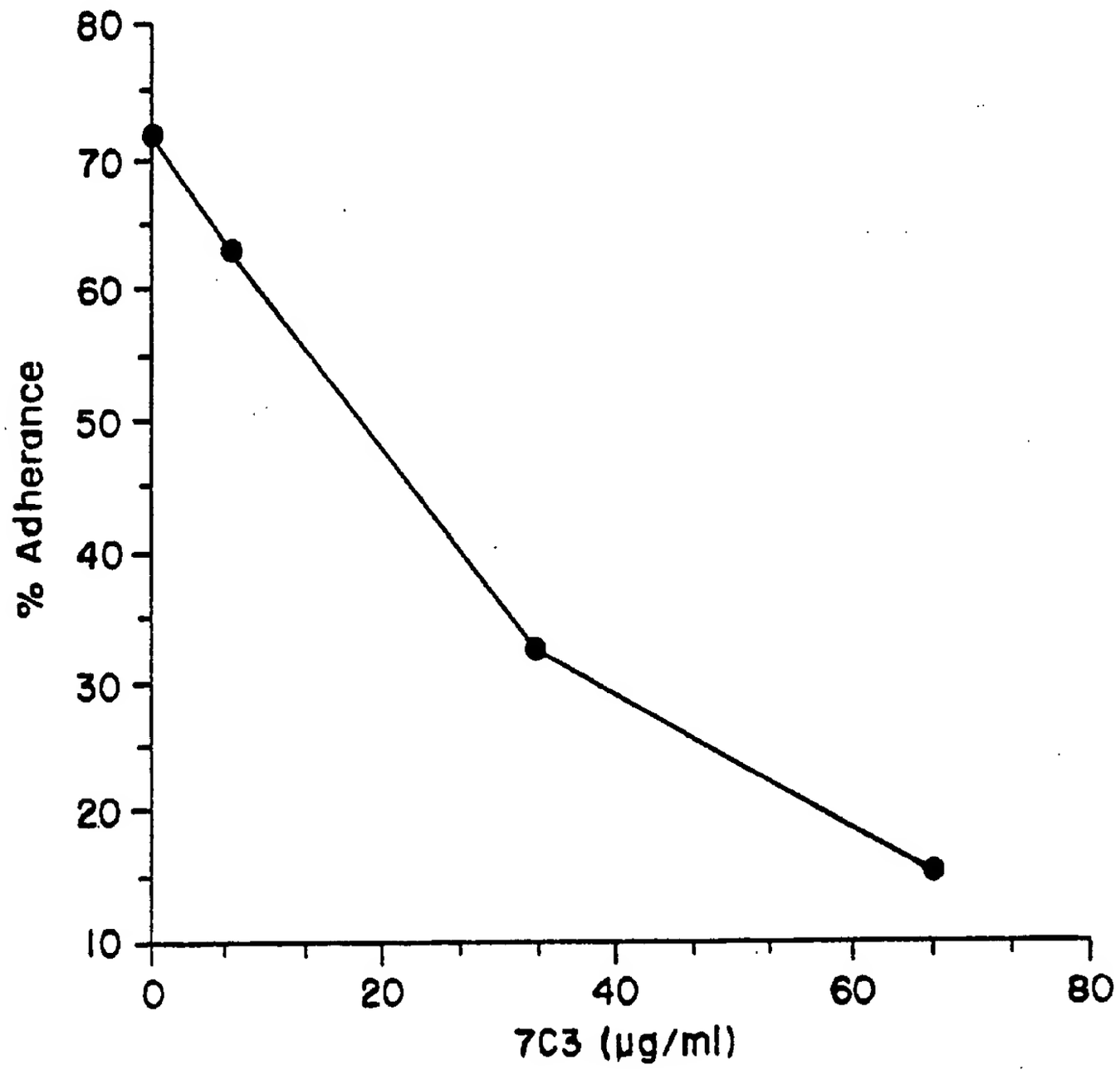

FIG. 3

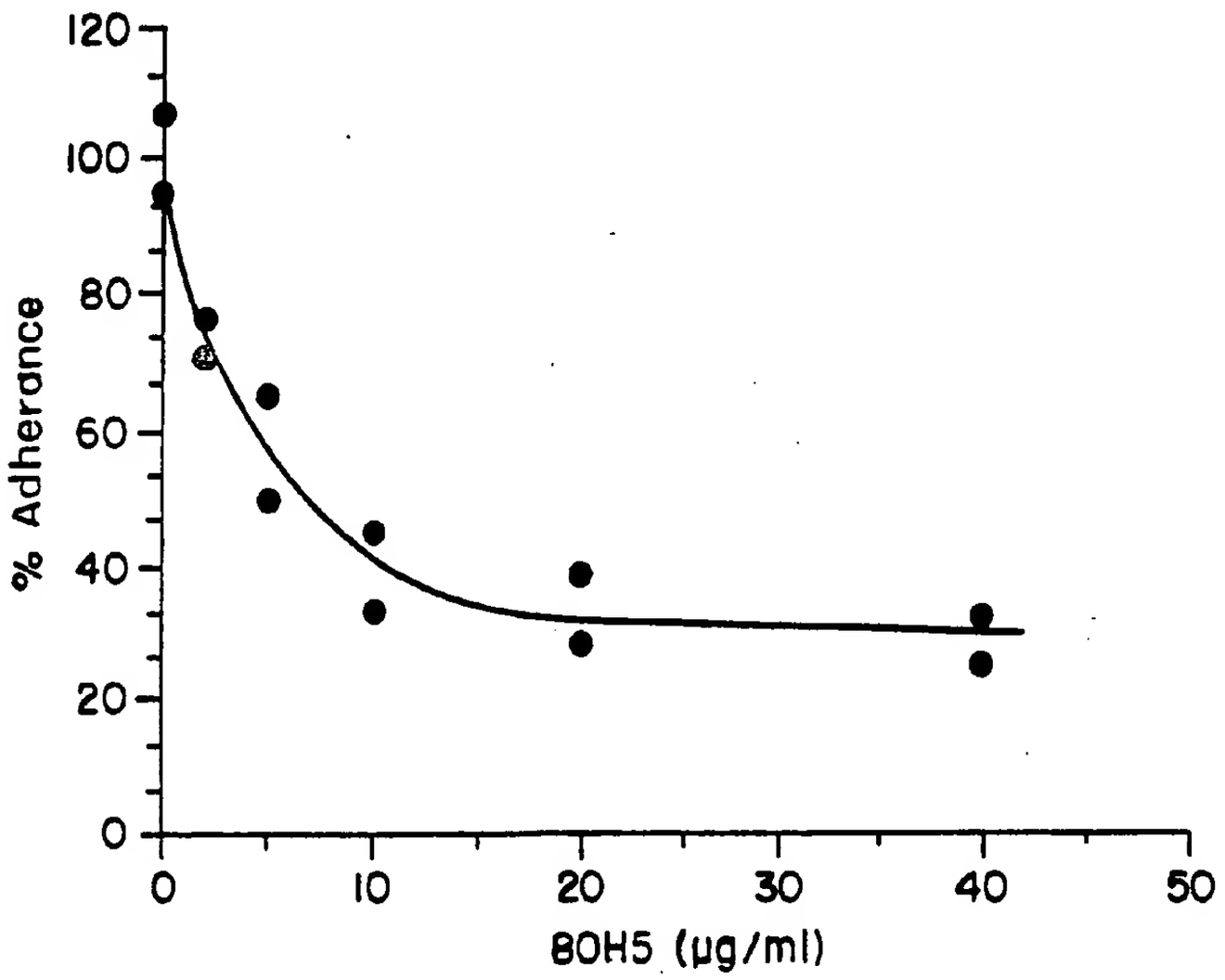

FIG. 4

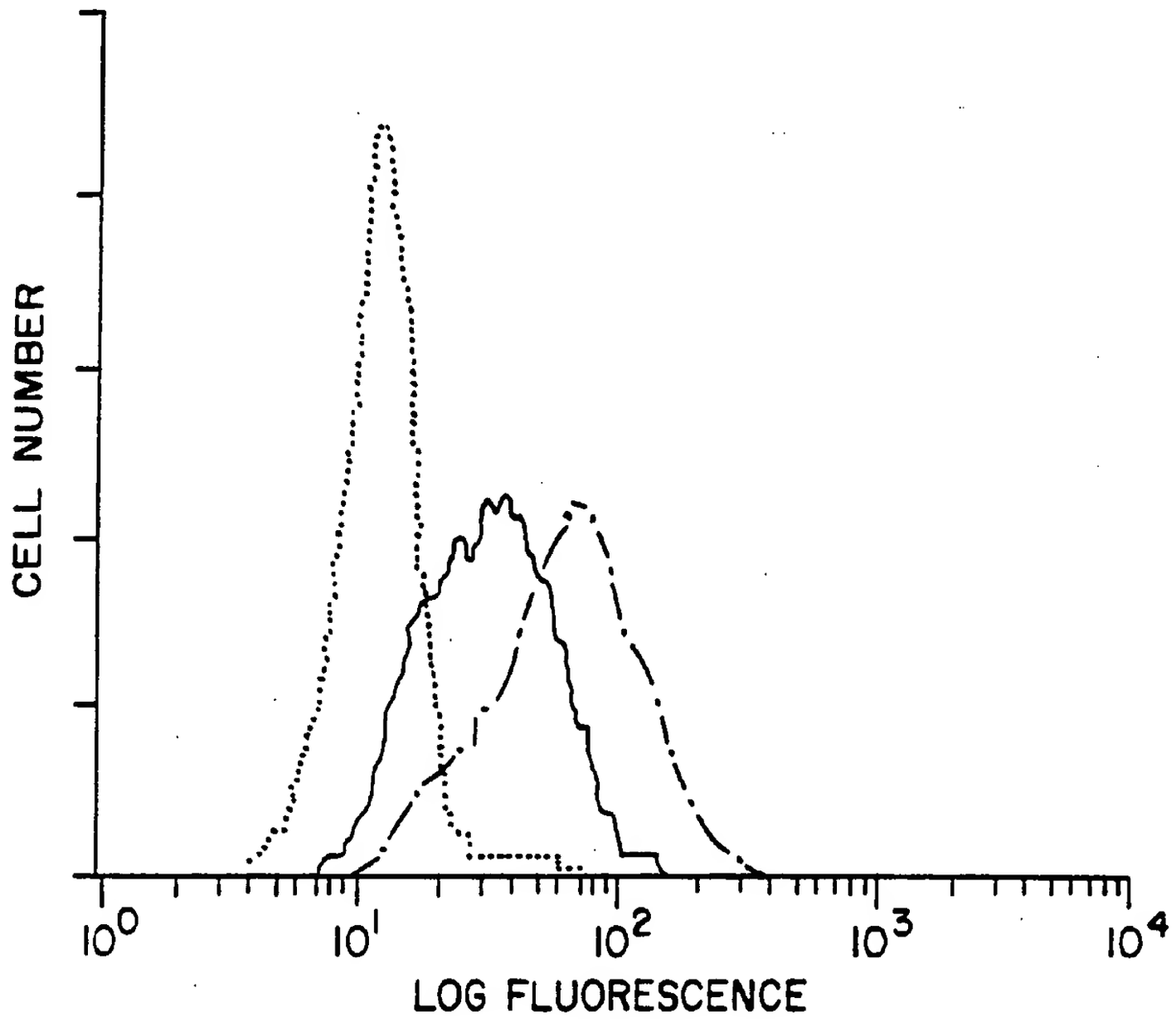

FIG. 5

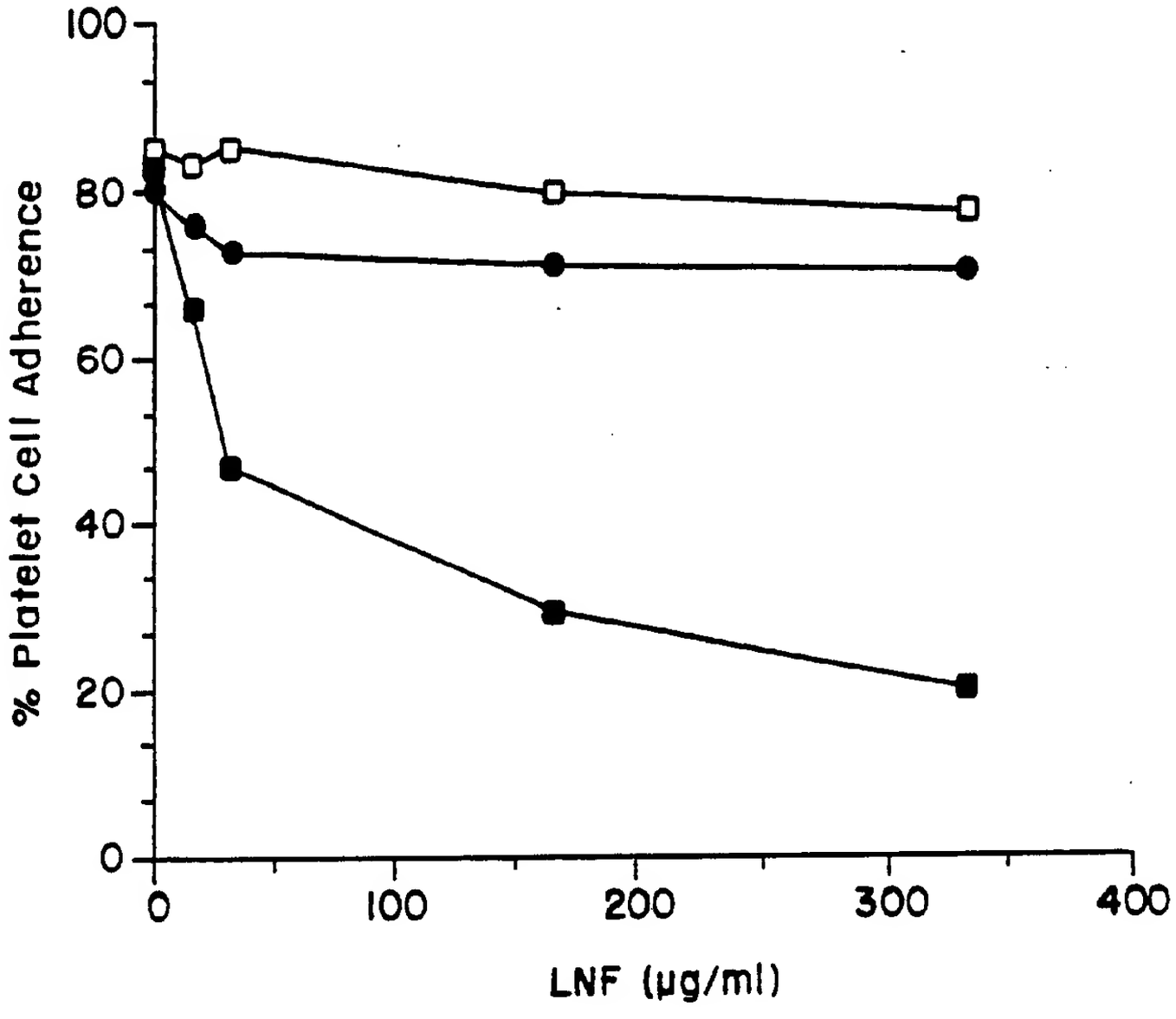
100
80
60
40
20
0
% Platelet Cell Adherence
0
100
200
300
400
LNF (µg/ml)

FIG. 6

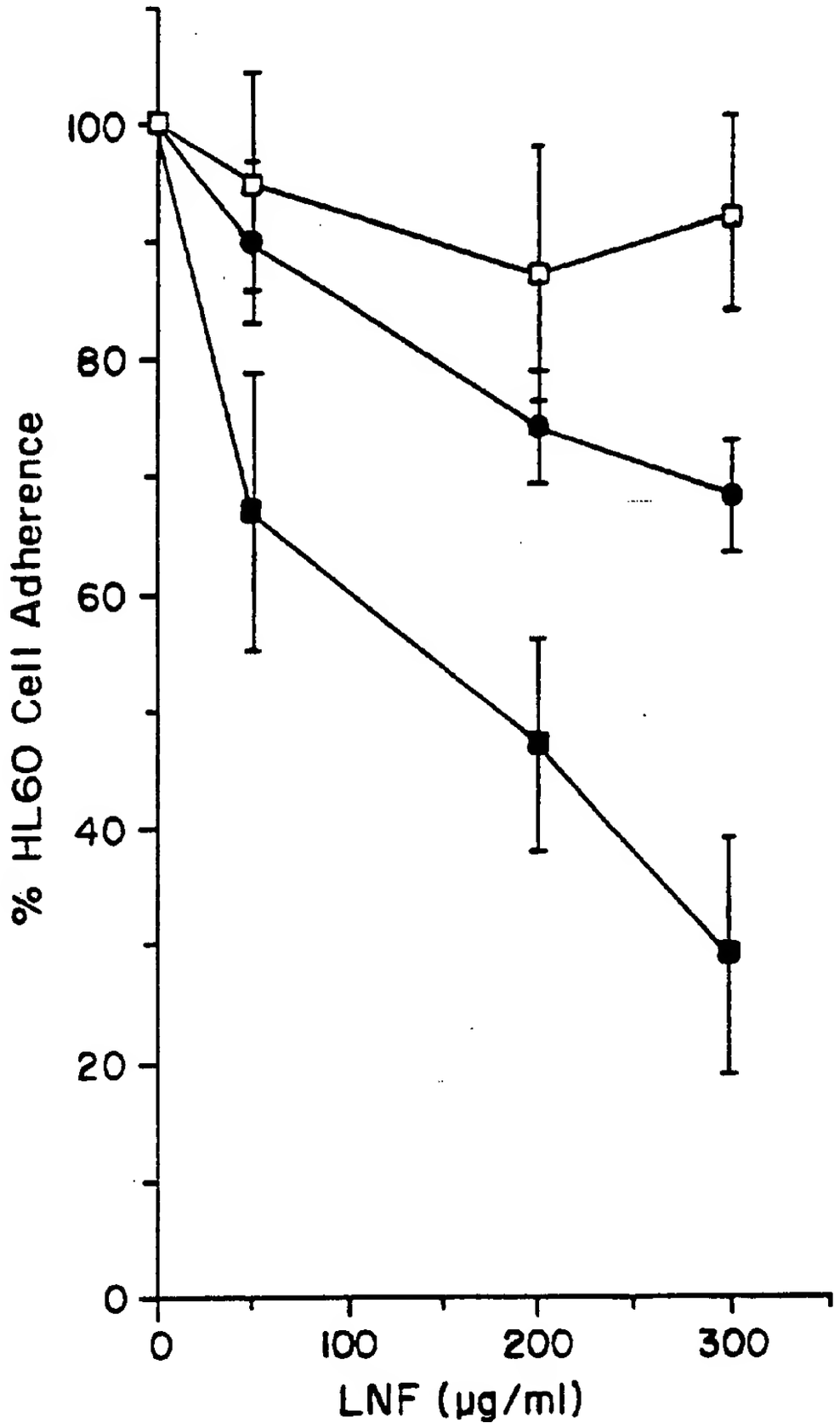

FIG. 7

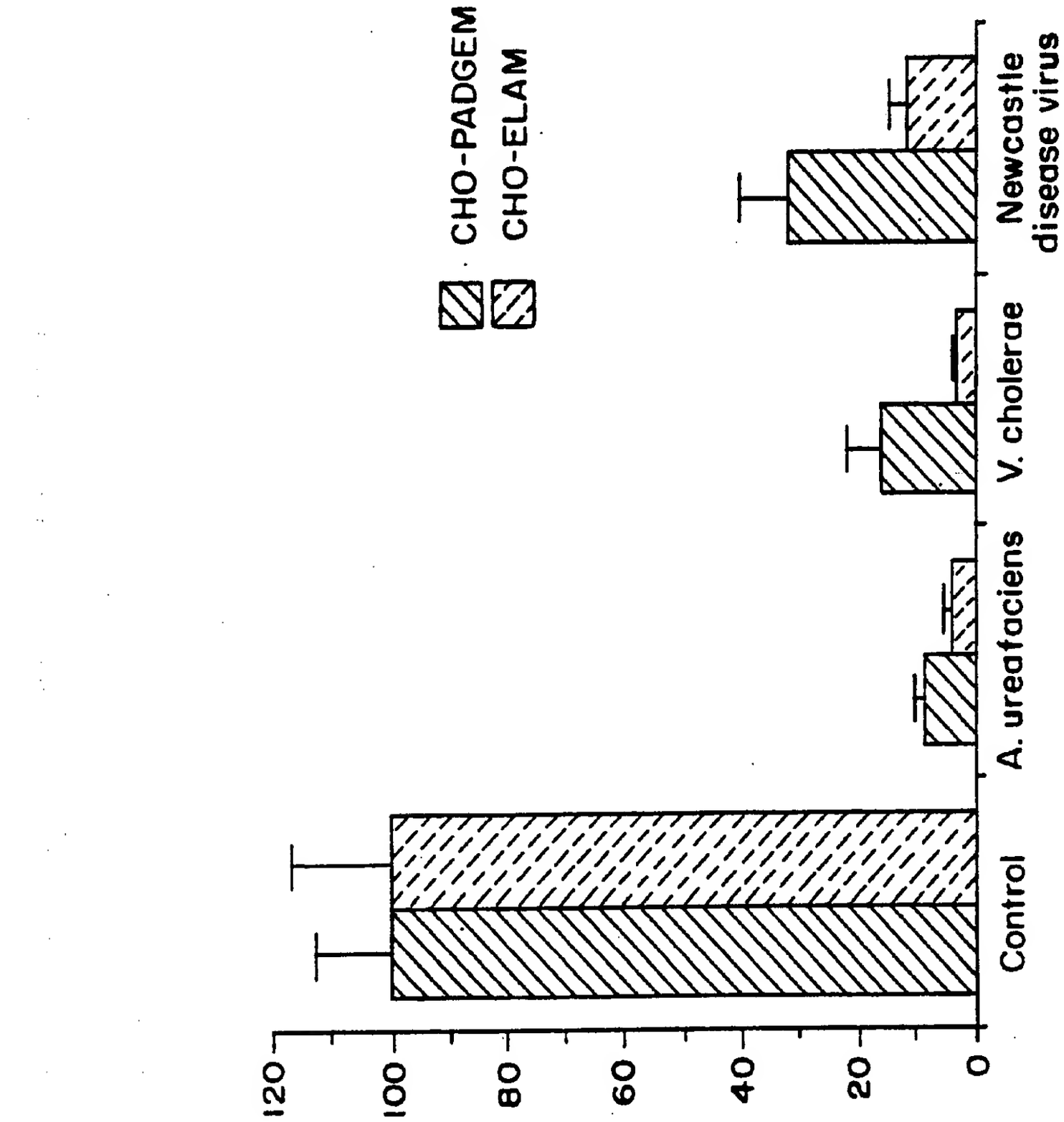
CHO-PADGEM
CHO-ELAM
Newcastle disease virus
Neuraminidase
V. cholerae
A. ureafaciens
Control
120
100
80
60
40
20
0
HL60 cell binding (% of control)

FIG. 8

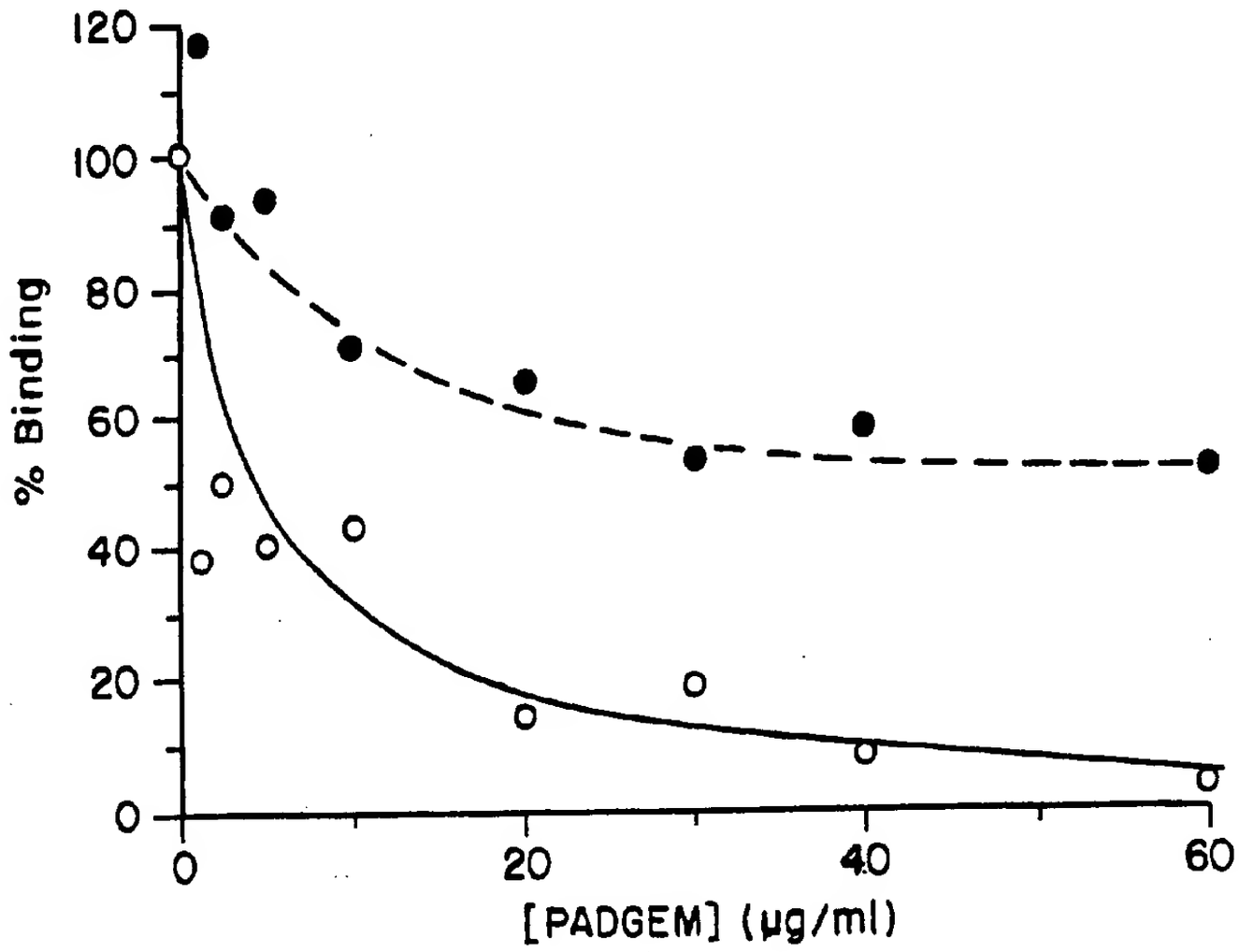

FIG. 9

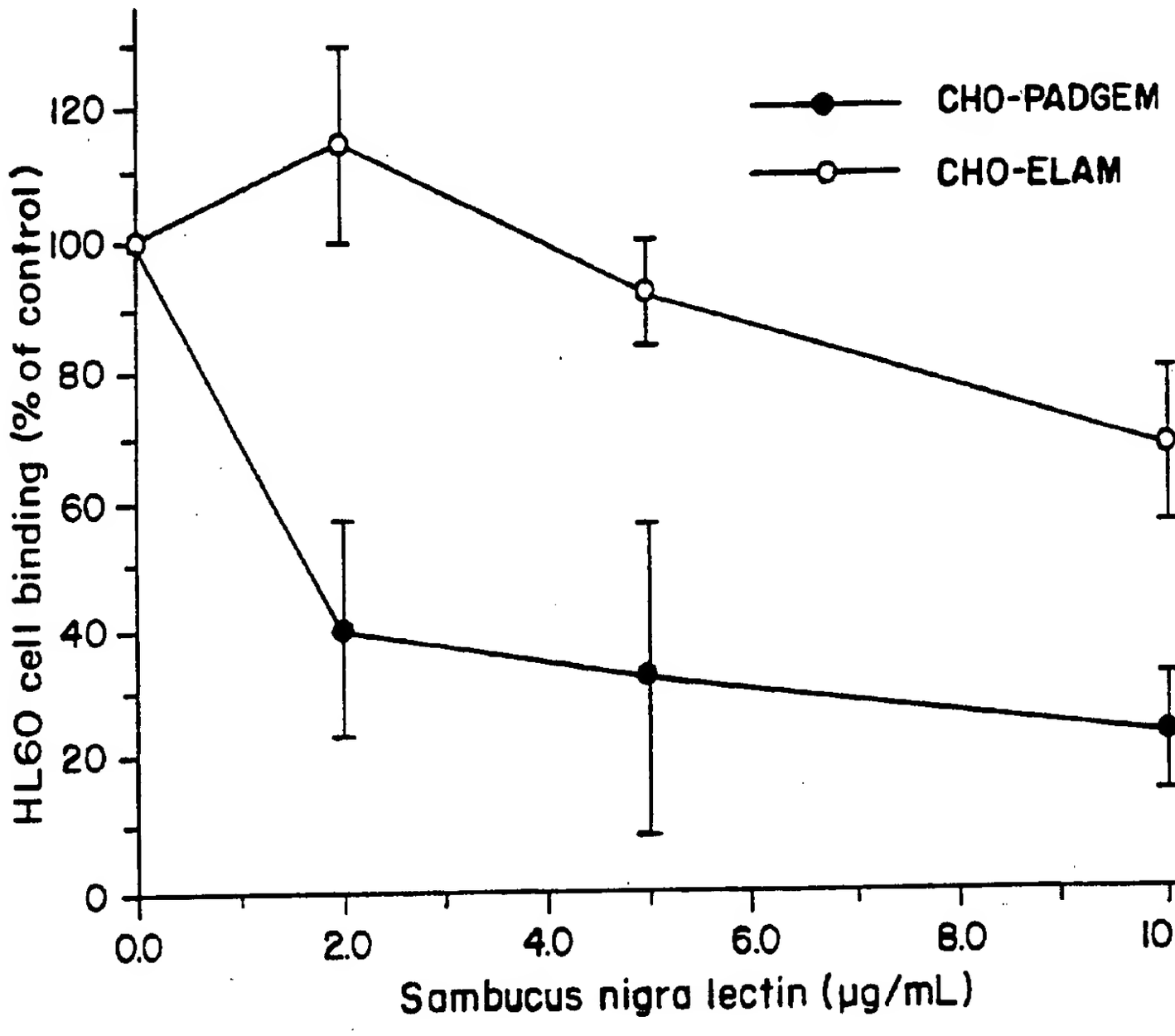
CHO-PADGEM
CHO-ELAM
HL60 cell binding (% of control)
0
20
40
60
80
100
120
0.0
2.0
4.0
6.0
8.0
10.0
Sambucus nigra lectin (µg/mL)

FIG. 10